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### Authors

Zhu, Q  
Zhang, M  
Rawlings, DJ  
et al.

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# Deletion within the Src Homology Domain 3 of Bruton's Tyrosine Kinase Resulting in X-linked Agammaglobulinemia (XLA)

By Qili Zhu,\* Min Zhang,\* David J. Rawlings,† Mauno Vihinen,§ Tracy Hagemann,¶ Douglas C. Saffran,‡ Sau-Ping Kwan,¶ Lennart Nilsson,§ C.I. Edvard Smith,|| Owen N. Witte,‡ Shi-Han Chen,\* and Hans D. Ochs\*

From the \*Department of Pediatrics, University of Washington, Seattle, Washington 98195; †Howard Hughes Medical Institute, University of California at Los Angeles, Los Angeles, California 90024-1662; §Center for Structural Biochemistry, and the ||Center for Biotechnology and Department of Clinical Immunology, Karolinska Institute, NOVUM, S-14157 Huddinge, Sweden; and the ¶Department of Immunology, Rush Medical College, Chicago, Illinois 60612-3833

## Summary

The gene responsible for X-linked agammaglobulinemia (XLA) has been recently identified to code for a cytoplasmic tyrosine kinase (Bruton's agammaglobulinemia tyrosine kinase, BTK), required for normal B cell development. BTK, like many other cytoplasmic tyrosine kinases, contains Src homology domains (SH2 and SH3), and catalytic kinase domain. SH3 domains are important for the targeting of signaling molecules to specific subcellular locations. We have identified a family with XLA whose affected members have a point mutation (g → a) at the 5' splice site of intron 8, resulting in the skipping of coding exon 8 and loss of 21 amino acids forming the COOH-terminal portion of the BTK SH3 domain. The study of three generations within this kinship, using restriction fragment length polymorphism and DNA analysis, allowed identification of the mutant X chromosome responsible for XLA and the carrier status in this family. BTK mRNA was present in normal amounts in Epstein-Barr virus-induced B lymphoblastoid cell lines established from affected family members. Although the SH3 deletion did not alter BTK protein stability and kinase activity of the truncated BTK protein was normal, the affected patients nevertheless have a severe B cell defect characteristic for XLA. The mutant protein was modeled using the normal BTK SH3 domain. The deletion results in loss of two COOH-terminal  $\beta$  strands containing several residues critical for the formation of the putative SH3 ligand-binding pocket. We predict that, as a result, one or more crucial SH3 binding proteins fail to interact with BTK, interrupting the cytoplasmic signal transduction process required for B cell differentiation.

X-linked agammaglobulinemia (XLA)<sup>1</sup>, first described by Bruton in 1952 (1), is characterized by markedly reduced or absent serum immunoglobulins of all isotypes and failure to produce antigen-specific antibodies (2, 3). The number of circulating B lymphocytes is drastically diminished, and germinal centers are absent in lymph nodes. X-chromosome inactivation studies have suggested that the gene defect

is intrinsic to the B cell lineage (3). The gene responsible for XLA, mapped by family studies to the long arm of the X chromosome at Xq22 (4), has recently been identified to be a cytoplasmic tyrosine kinase (Bruton's agammaglobulinemia tyrosine kinase, BTK) (5–7). The enzyme, BTK, is expressed in most hematopoietic cells, but is selectively down-regulated in plasma cells and T lymphocytes (5, 6, 8, 9) and appears to play a critical role in the regulation of B cell proliferation and differentiation. Like most other cytoplasmic tyrosine kinases, BTK contains SH2 and SH3 regions (Src homology domains), and a catalytic kinase domain (10). Unlike Src subfamily cytoplasmic tyrosine kinases, BTK lacks a COOH-terminal regulatory tyrosine and an NH<sub>2</sub>-terminal myristylation signal. A recently identified novel domain, the pleckstrin homology (PH) domain present in many signaling proteins, is also found in the NH<sub>2</sub>-terminal region of BTK

<sup>1</sup> Abbreviations used in this paper: B-LCL, B lymphoblastoid cell line; BTK, Bruton's agammaglobulinemia tyrosine kinase; Kv, K value (rate of bacteriophage inactivation); PH, pleckstrin homology; PI-3 kinase, phosphatidylinositol-3' kinase; RT, reverse transcriptase; SH, Src homology domain; TH, Tec homology; xid, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia.

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(9, 11). These features, as well as the existence of a conserved 60–80 amino acid segment located between the PH and SH3 domains, tentatively designated the Tec homology (TH) domain (9), have suggested BTK and several other homologous, lineage-restricted tyrosine kinases are members of a different kinase subfamily (5, 9, 11).

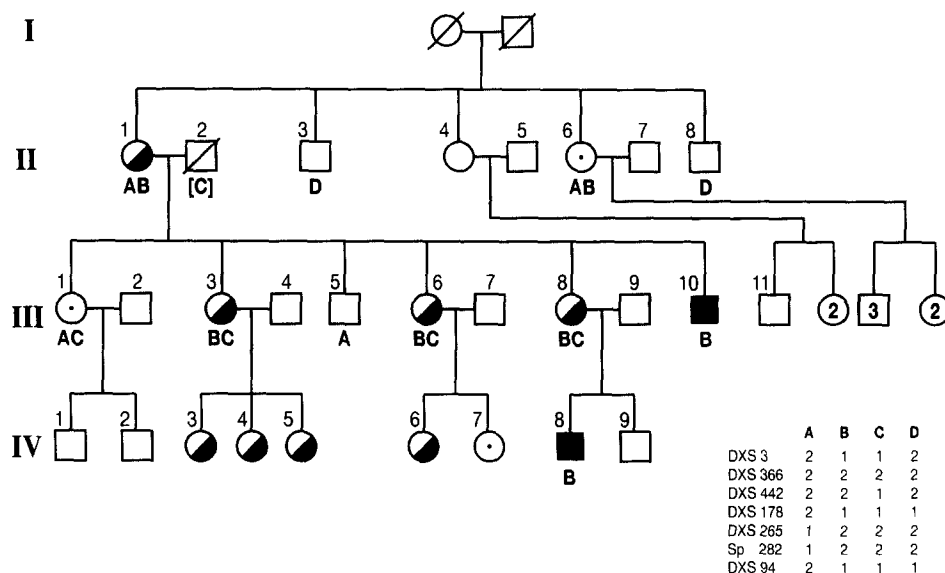
The initial reports implicating BTK in XLA demonstrated loss of BTK kinase activity (5) or point mutations resulting in single amino acid substitutions within the kinase (SH1) domain (6). Pre B and B cell lines established from XLA patients were found to have reduced or absent *BTK* mRNA, BTK protein expression, and kinase activity (5). Subsequently, additional substitutions and stop codon formation have been reported in this domain (12, 13).

These studies demonstrated a critical role for BTK in early B cell development. Identification of mutations within other BTK functional domains, however, is likely to lead to additional insights into the BTK signaling pathways. Two such informative mutations have recently been described, both in the PH and in the SH2 domains. A point mutation within the *Btk* PH region affecting residue 28 (Arg → Cys) results in a moderately severe X-linked immunodeficiency (xid) in the mouse (14, 15), whereas severe XLA was found in a family with four affected brothers having the same residue mutated to His (13). This mutation alters a highly conserved residue within the (PH) domain of BTK (9, 11, 16). Unlike the R28H mutation in the XLA patients, the R28C mutation in the xid mouse affects B cell signaling without blocking pre-B cell clonal expansion. Mutations in the SH2 domain were found in both “atypical” and “classical” (severely affected) XLA patients (12, 13, 17). These mutations altered highly conserved residues (R288W; R307G; Y361C) important in forming the SH2 phosphotyrosine binding pocket. The mutant BTK protein (Y361C) isolated from a B cell line established from a patient with atypical XLA had decreased stability predicted to result from the alteration in SH2 binding function (17).

In the present study, we report a point mutation at a donor splice junction, resulting in exon skipping and loss of 21 amino acids forming the COOH-terminal portion of the BTK SH3 domain. The SH3 domain is a small (60–80 amino acid) module present in a large number of proteins involved in signal transduction and membrane cytoskeletal interactions. The function of the SH3 domain is unknown, but it binds to peptides rich in proline and hydrophobic residues (18–20). The structure has been determined for five SH3 domains: Src (21), Fyn (22), spectrin (23), p85 of phosphatidylinositol-3' kinase (PI-3 kinase) (24, 25), and PLC $\gamma$  (26). These all share the same structural scaffolding where insertions and deletions appear on loops connecting adjacent  $\beta$ -strands. The putative binding site lies on one face of the domain and is predicted to be significantly altered by this deletion (23). Despite the deletion, the truncated BTK protein is stable and remains functionally active. However, the deletion results in the XLA phenotype in two affected males, suggesting that the SH3 domain is likely to play a critical role in BTK-dependent signaling and normal B cell development. To our knowledge, this is the first naturally occurring mutation within, and limited to, an SH3 domains resulting in a human genetic disease.

## Materials and Methods

**XLA Family.** The members of a large family with an affected uncle/nephew pair are shown in Fig. 1. Patient III-10 is a 41-yr-old male who had recurrent bacterial infections since early infancy, resulting in chronic otitis media, chronic sinusitis, and bronchiectasis. Before treatment with Ig was initiated, he had severe arthritis involving multiple joints; serum IgG concentration was 135 mg/dl; IgA and IgM were not detectable. When immunized with bacteriophage  $\phi$ X174 (27), he had prolonged circulation of phage and failed to produce phage neutralizing antibody. His peripheral blood B lymphocyte proportion has been consistently <0.1%. A rectal biopsy showed absence of plasma cells. His nephew (IV-8) is an 11-yr-old boy who at 6 mo of age, when the diagnosis was estab-



**Figure 1.** Pedigree of a family with XLA. The two affected males (III-10 and IV-8) are indicated by solid symbols (■). Carrier females, as determined by RT-PCR and by amplification mutagenesis, are identified by half-filled symbols (◐); (◑) indicate female members at risk studied in similar fashion and found to be nonaffected. Symbols A, B, C, and D indicate haplotypes as defined by RFLP and markers linked to the *BTK* locus; these markers are described in detail in the text. The alleles at each locus are indicated with a 1 for the smaller and a 2 for the larger allele. The genotype for individual II-2 is deduced.

lished and before treatment with intravenous immunoglobulin (IVIG) was initiated, had a serum IgG level of 267 mg/dl; IgA and IgM were not demonstrable. He had <1% B cells. When immunized with bacteriophage, patient IV-8 cleared antigen within 1 wk and showed a low but definite antibody response (patient's peak K-value [Kv, rate of bacteriophage inactivation] post primary immunization was 0.029 [normal Kv = 50.1; 95% confidence interval, 3.2–807]; patient's peak Kv post secondary immunization was 0.43 [normal Kv = 362; 95% confidence interval, 119–1037]). All of this antibody was of the IgM class. He responded well to IVIG therapy and is presently asymptomatic and without chronic disease. The mothers (II-1 and III-8) of the two patients are healthy. II-1 had four healthy brothers, two still alive, and two sisters who have between them four healthy boys.

**Cells and Cell Lines.** PBMC were separated from heparinized venous blood by centrifugation over lymphocyte separation medium (Ficoll-Paque; Pharmacia LKB, Uppsala, Sweden). EBV-transformed B lymphoblastoid cell lines (B-LCL) were derived from PBMC infected with supernatants from the marmoset cell line B95.8 (28). B-LCL were established from both patients (III-10, IV-8) and from the obligate carriers (II-1, III-8).

**DNA Purification, Southern Analysis, and RFLP.** Genomic DNA was extracted from leukocytes of 5 ml whole blood (anticoagulated with EDTA) and purified using a method described in the Quiagen Genomic Handbook (provided by Quiagen Inc., Chatsworth, CA).

Purified genomic DNA from the XLA patients and normal controls was digested overnight separately with restriction enzymes EcoRI, HindIII, and TaqI. The digested DNA samples were subjected to agarose (0.8%) gel electrophoresis and the DNA fragments were transferred from the gel to a nylon membrane, followed by hybridization with a <sup>32</sup>P-labeled BTK full-length cDNA probe 14-6 (6).

Markers used to analyze the inheritance pattern of alleles in the region of q21.3-q22 on the X chromosome included probes for DXS366, DXS442, and DXS265 (gift of Dr. David Barker, University of Utah, Salt Lake City, UT) (29), DXS3 (gift of Dr. Gail Bruns, Boston Children's Hospital, Boston, MA) (30), SP282 (31); DXS94 (32) and DXS178 (33) were obtained from American Type Culture Collection (Rockville, MD). DNA, prepared from EDTA blood collected from selected family members (Fig. 1), was digested to completion with the appropriate restriction enzyme for each marker (TaqI for DXS366, DXS3, and DXS178, MspI for DXS442 and DXS265, BstNI for SP282, and PstI for DXS94) and Southern blotted for hybridization to the appropriate probes as previously described (34).

**PCR and Sequencing of Genomic DNA.** Purified genomic DNA samples, prepared from controls and members of the XLA family, were amplified with two amplification primers, 5'-CAGGAAGGC-TACATTCCT-3' and 5'-CTCCGAGTCATGTGTTTGA-3', which span the suspected mutation site and whose selection was based on the published cDNA sequence (6); the former primer corresponded to nucleotides 910–927, the latter was complementary to nucleotides 998–979 of the cDNA sequence. The following PCR conditions were used: 5 min at 94°C followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The resulting PCR products were ~1,300 bp in length. The amplified DNA fragments were separated by agarose gel electrophoresis and electroeluted. Direct sequencing was performed by the dideoxynucleotide chain termination method (35), using the sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, OH).

**Amplification Mutagenesis.** Amplification mutagenesis is a PCR method designed to detect genomic mutations by directly screening

DNA fragments. A new restriction enzyme site is created by introducing (or changing) one nucleotide near the 3' end of an amplification primer. After regular amplification, the new site can be recognized (either the normal or the mutant sequence but not both) by a designated enzyme (36). The set of primers we designed for the amplification mutagenesis to detect the mutation were the "sense" primer 5'-TGAATCTGTCCTCGAGGC-3' and the "anti-sense" primer, 5'-GTGGACTGACATAACATACGTA-3' which served as mutagenesis primer having one base substitution (G); this created a new restriction enzyme RsaI site for the amplified DNA. The resulting DNA fragment consisted of 149 bp. After RsaI digestion, the fragments were electrophoresed in a 2% Metaphor TM agarose gel (FMC Bioproducts, Rockland, ME) and the DNA bands were made visible with ethidium bromide staining.

**RNA Isolation and Northern Blot Analysis.** Total RNA was isolated from PBMC or B-LCL obtained from normal controls and from members of the XLA family, using a single step method and Trizol™ (GIBCO BRL, Gaithersburg, MD) (37). Approximately 10 µg of total RNA was electrophoresed on a formaldehyde-agarose gel, transferred to nylon membrane and hybridized with a <sup>32</sup>P-labeled BTK cDNA clone 14-6 (6), as previously described (38).

**Reverse Transcriptase (RT)-PCR and Sequencing.** First-strand cDNA was prepared from RNA isolated from PBMC or B-LCL obtained from normal controls and members of the XLA family. 2.5 µg of total RNA was incubated with oligo (dT) as primer using the SuperScrip Preamplification System kit (GIBCO BRL) as recommended by the manufacturer. PCR was performed using a modified method described by Saiki et al. (39) with an automatic DNA thermal cycler (Coy Laboratory Products Inc., Ann Arbor, MI). The PCR reactions underwent 35 cycles at 94°C for 2 min, 60°C for 1.5 min, 72°C for 2 min, and a final extension step at 72°C for 10 min. Overlapping primer pairs were selected from the known BTK cDNA sequence to cover the entire coding region. The amplified DNA fragments were isolated by agarose gel electrophoresis; the bands were made visible with ethidium bromide and in selected cases electroeluted. Direct sequencing of the eluted material was performed using a modified dideoxynucleotide chain termination method (40) and the PFU DNA sequencing kit (Stratagene, La Jolla, CA).

**BTK Protein Analysis.** To measure BTK protein synthesis, 10<sup>7</sup> EBV transformed B lymphoblasts were incubated for 4 h with 250 µCi [<sup>35</sup>S]methionine/ml in methionine-free medium. The labeled protein was immunoprecipitated with anti-BTK antibody, as described previously (5). Immunoprecipitates were electrophoresed by SDS-page and visualized by autoradiography. To determine autokinase activity, BTK was immunoprecipitated with anti-BTK antibody from lysates of patient and normal B lymphoblasts, and activity assayed by addition of γ-[<sup>32</sup>P]ATP in 20 mM PIPES (pH 7.0) containing 20 mM MnCl (5). Samples were run on SDS-page and visualized by autoradiography. To determine transphosphorylation of exogenous substrate, immunoprecipitated BTK from normal and patient B-LCL lysates was incubated in the presence of acid-denatured enolase and γ-[<sup>32</sup>P]ATP (5).

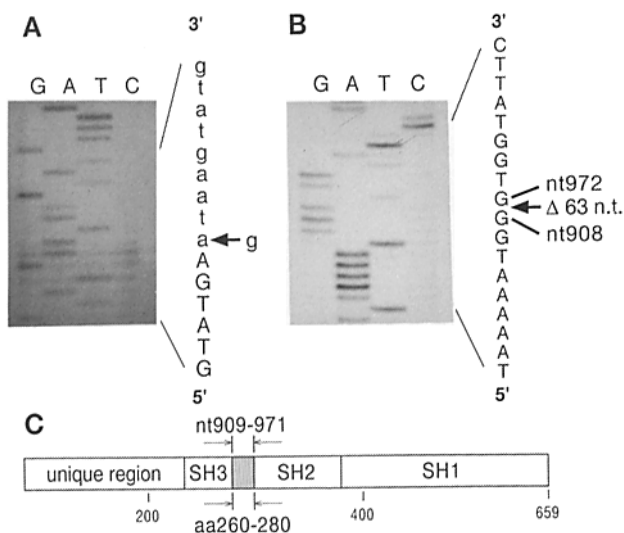
**Computational Techniques.** The BTK SH3 sequence was compared with those of SH3s for which three-dimensional structures have been determined and were available, including Fyn, spectrin, and p85 of PI-3 kinase. The BTK SH3 structure was modeled with InsightII (Biosym Technologies, Inc., San Diego, CA) and refined by energy minimization with the program CHARMM (41). Minimization was performed stepwise with adopted basis Newton-Raphson algorithm first relaxing hydrogen atoms. Then harmonic constraints were put on the conserved regions and the loops were minimized for 1,000 steps after which C<sub>α</sub> atoms in the conserved

region were harmonically constrained. The SH3 deletion was made by discarding the 14 COOH-terminal residues (the other seven deleted amino acids are in a loop joining SH3 and SH2 domains) from the SH3 model and performing the minimizations described above.

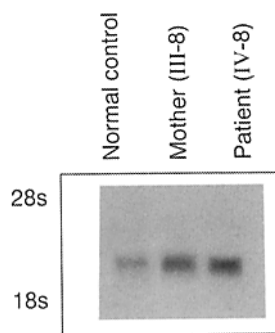
Both the normal BTK SH3 and the deletion mutant were subjected to molecular dynamics at 300 K with the CHARMM program. The minimized structures were used in the simulation in vacuo. The parameters used in the energy function were standard CHARMM parameters. The molecular dynamics integrations were done using the time-step of 1 fs at a constant temperature of 300 K. The system was slowly warmed to 300 K over the course of 8.5 ps. After the warming period, the simulation was continued for an additional 188.5 ps. The temperature was checked every five time-steps and allowed to vary 10 K. The SHAKE routine (42) was used to constrain covalent bonds between hydrogens and heavy atoms.

## Results

**A Splice Donor Mutation Results in Exon Loss of BTK.** We performed Southern blot analyses of patient genomic DNA digested with restriction enzymes EcoRI, HindIII, and TaqI, and hybridized to the *BTK* probe 14-6. No major differences in restriction DNA fragment sizes were identified when those of the patients were compared with those of normals (results not shown). Direct sequencing of amplified genomic DNA fragments revealed a g → a point mutation involving the first nucleotide of a donor splice junction in both patients' *BTK* genes (Fig. 2 A). The mutation involves a critical nucleo-



**Figure 2.** PCR-sequence analysis of genomic DNA and cDNA of *BTK* of the affected males. (A) Location of the g → a point mutation involving the first nucleotide of a donor splice junction at the 5' end of an intron. This mutation alters the splice donor site 5'-gtaagt to ataagt, preventing the pairing and exon joining at the 3' site. (B) Sequence analysis of patient cDNA indicate the deletion of 63 nucleotides between nucleotide 908 and 972. (C) The deletion of 63 base pairs coding for amino acid residues 260–280, caused by the g → a point mutation involving the first nucleotide of the donor splice junction is indicated. The deletion, located in the cDNA coding for the COOH-terminal portion of the SH3 domain, involves nucleotides 909–971.



**Figure 3.** Normal expression of *BTK* mRNA in B cells from the XLA patients. mRNA was isolated from B-LCLs established from a normal control, a carrier female (III-8), and an XLA patient (IV-8). The mRNA obtained from the XLA patient is smaller than control mRNA.

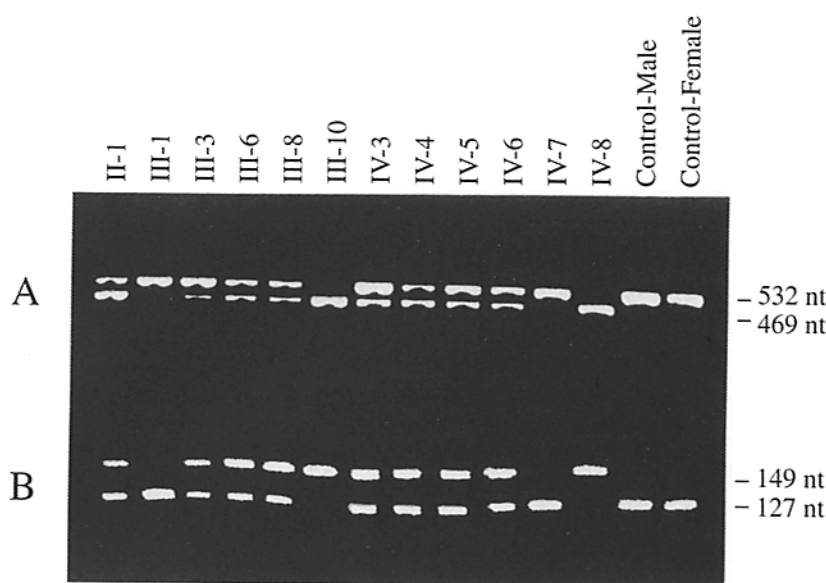
tide at the 5' end of an intron required for proper splice function (43).

Northern blot analysis of total RNA isolated from PBMC and B-LCLs of the two patients and the two obligate carrier females (II-1, III-8) demonstrated the presence of normal quantities of mRNA; Fig. 3 illustrates mRNA isolated from B-LCL of a normal control, a carrier female, and a patient. The patient's mRNA appears slightly smaller than control mRNA.

*BTK* cDNA was generated and amplified by RT-PCR, using two overlapping primer pairs that covered the entire coding region of *BTK*. The PCR amplified cDNA fragments spanning nucleotides 763–1294, derived from the cDNA of XLA patients III-10 and IV-8 (using primers GTCTCCACA-AGTGAGCTGAA [sense] and CAGTGGGAAGGTGCAT-TCTTG [antisense]), were shorter than those obtained from normal controls (Fig. 4 A). Sequencing of the PCR products revealed that the patients' cDNA had a deletion of 63 nucleotides including bp 909 and 971 (Fig. 2, B and C). To exclude a PCR artifact, we used two separate RNA preparations isolated from B-LCLs derived from each of the two patients for RT-PCR and found the same deletion each time.

**Carrier Detection.** To trace the inheritance and possible origin of the affected X chromosome, we selected seven markers closely linked to the region of the *BTK* locus (Xq21.3–Xq22) to be used in RFLP analysis. The polymorphisms at DXS3, DXS442, DXS178, DXS265, SP282, and DXS94 indicated that individuals III-3, III-6, III-8, and III-10 had inherited from their mother (II-1) the same marker alleles (haplotype B in Fig. 1) linked to the *BTK* locus. This data identifies III-3 and III-6 as possible carriers, since their brother III-10 is affected with XLA. Assuming that no crossovers between the marker alleles and *BTK* occurred, individual III-1 is not a carrier; she inherited the same maternally derived allele (allele A in Fig. 1) as her normal brother III-5. Further analysis shows that individual IV-8 who was diagnosed with XLA (and his obligate carrier mother III-8) also inherited the B haplotype associated with the defective *BTK* gene.

To identify accurately those females carrying the X-chromosome with the point mutation located within the *BTK* gene, we developed a simple detection method using amplification mutagenesis and compared the results with those obtained by RT-PCR and electrophoresis. A DNA fragment of 149 bp was selected for amplification mutagenesis. When generated

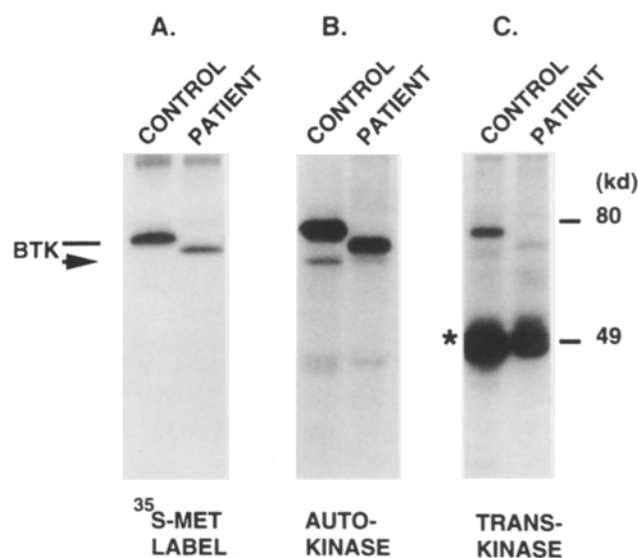


into two segments, one being 127 bp and the other (too small to stay on the gel) 22 bp. In contrast, DNA fragments with the  $g \rightarrow a$  mutation cannot be digested by *RsaI*. The affected males are identified by a DNA fragment size of 149 bp. The eight female carriers showed two bands, one normal (127 bp) and the other abnormal (149 bp). The results of the two techniques are in complete agreement.

from genomic DNA of individuals with normal BTK, this fragment is cleaved by restriction enzyme *RsaI* into two segments, one being 127 bp and the other 22 bp; the latter is too small to show up on the gel. However, DNA with the  $g \rightarrow a$  mutation cannot be digested by *RsaI*. The DNA fragment size (Fig. 4 B) identifies the affected males who have a single band of 149 bp and the eight female carriers with two bands, one normal (127 bp) and one abnormal (149 bp). The results are in agreement with analysis of PCR amplified fragments spanning nucleotides 763–1294 derived from cDNA of female members of the XLA family (Fig. 4 A).

**Mutant SH3 BTK Results in XLA without Affecting Kinase Activity.** The amount of BTK protein synthesized by B lymphoblasts derived from patient III-10 was estimated by [ $^{35}$ S]methionine metabolic pulse labeling. Protein expression was quantitatively similar to that of control B lymphoblasts (Fig. 5 A). This is consistent with the normal expression of BTK mRNA shown in Fig. 3 and suggests that the SH3 deletion does not significantly alter the BTK protein stability. As predicted by sequence analysis, the BTK protein product of the patient's B-LCL was truncated. To assess the function of the altered BTK, we measured phosphorylation activity in vitro. Both autophosphorylation (Fig. 5 B) and transphosphorylation of the exogenous substrate, enolase, (Fig. 5 C) by the mutant BTK was equivalent to control BTK, indicating that functional kinase activity of the truncated BTK is normal.

**Structure of the BTK SH3 Domain.** The BTK SH3 sequence was compared with those of Fyn, spectrin, and p85 of PI-3 kinase, for which structural coordinates were available. The



**Figure 5.** Expression of BTK by B lymphoblasts from an affected male and a normal control. (A) B lymphoblasts were incubated with [ $^{35}$ S]methionine in methionine-free medium. BTK was immunoprecipitated with anti-BTK antibody, electrophoresed by SDS-PAGE, and autoradiographed. BTK protein expression by B lymphoblasts from patient III-10 was similar to that of control B lymphoblasts (horizontal line), but the size was smaller ( $\rightarrow$ ), reflecting the deletion of 21 amino acids. Incorporation of metabolic label, measured by TCA precipitable counts, was 40% lower in the mutant B lymphoblasts and accounts for the reduction in signal in the patient lane (B) BTK function as measured by autophosphorylation using [ $^{32}$ P]ATP was similar in the patient and in the control; the patient BTK was truncated. (C) Transphosphorylation of exogenous substrate (Asterisk, enolase) was similar in the immunoprecipitated BTK from patient and control B lymphoblasts.





Src family member Fyn was found to have the greatest identity, 45% (Fig. 6 A), and was thus used as the template for modeling. There was only a single deletion of one residue on a short loop connecting two  $\beta$  strands. Most of the amino acid substitutions were by physicochemically related ones, and the sequences were very related facilitating reliable modeling.

The structure of the normal BTK SH3 consists presumably of two antiparallel  $\beta$ -sheets (Fig. 6) and is very similar to Fyn and Src both at the sequence and structural level. The single amino acid deletion in wild type BTK (as compared with wild-type Fyn) did not change the fold. This loop is known to have the largest deviations in Fyn when compared with other SH3s (21). The central hydrophobic core is conserved also in BTK; of the six residues in Fyn, four are identical in BTK, and one (F109 in Fyn) is replaced by another aromatic residue. SH3 of Fyn and Lyn were recently shown to bind to a proline-rich region within the 85-kD subunit (p85) of PI-3 kinase, leading to a five- to sevenfold increase in the specific activity of PI-3 kinase (20). The ligand binding of Src has been studied with short proline-rich peptides (21). Many of the Src residues perturbed by ligand binding are either identical or substituted by related amino acids in BTK (Fig. 6 A).

The SH3 deletion mutant was modeled from the normal BTK SH3. This structure is missing the 14 COOH-terminal residues including the two COOH-terminal  $\beta$  strands and the helix (Fig. 6). Thus the deletion mutant is lacking most of the residues forming the putative ligand binding region and missing essential parts of both the  $\beta$ -sheets (Fig. 6 B). When substantial parts of a protein are missing, its conformation could be drastically altered. To analyze if this were the case in the BTK SH3 deletion mutant, the conformation was studied with modeling and molecular dynamics. The mutant was found to have a stable structure, although somewhat different from the fold of corresponding section in the normal SH3. The backbone atoms did not fluctuate more than in globular proteins in general, with a root mean square fluctuation of 0.64 Å during the last 120 ps of simulation, indicating stable structure. The organization of the secondary structural elements are shown in Fig. 6 B. The flexible NH<sub>2</sub> and COOH termini are separated by 12–15 Å, while in the wild type BTK SH3, the calculated distance is 13–14 Å.

## Discussion

The recent identification of the gene responsible for XLA allowed us to study the genetic defect and its effect on the function of BTK in a large family with two affected males. Both patients were found to have demonstrable B cells in the peripheral blood that could be immortalized by EBV, and one patient (IV-8) was able to clear bacteriophage and to produce a small amount of antibody (<1% of normal). These findings suggest a milder variant of the disease (44) although both patients fulfill the criteria for XLA.

The abnormality of the BTK gene responsible for the XLA phenotype in this family is a g  $\rightarrow$  a point mutation at the 5' end of intron 8 (intron assignment is based on our unpublished data). This mutation alters the splice donor site

5'-gtaagt to ataagt. The first critical step during RNA processing is pairing of the 5' splice site consensus sequence (5'-gtaagt) with the complementary sequence on the small nucleoribonuclear protein, U1 snRNA, within the spliceosome (43). The g  $\rightarrow$  a mutation at the 5' splice site of intron 8 alters an invariant nucleotide in the consensus sequence, preventing the pairing and exon joining at the 3' site (45) and thus causes the skipping of coding exon 8, the most COOH-terminal of the exons forming the BTK SH3 domain. In a recent review of 29 missense mutations involving the invariant g nucleotide at the 5' splice sites of genes causing various human diseases, 11 mutations were identified whose only consequence was skipping of an exon (46). In the remaining mutations, exon skipping was associated with alternative splicing at nearby sites. In the XLA patients described here, coding exon 8 was skipped, causing in frame splicing of exon 7 to exon 9, and deletion of 63 nucleotides. The resulting shortened mRNA translated into a truncated protein with a 21 amino acid deletion (residues 260–280) within the SH3 domain and the loop joining SH3 and SH2 of the BTK protein (Fig. 2 C). This prediction was confirmed by sequence analysis of cDNA and by Western blot analysis of the BTK protein.

The availability of three generations from this family for study allowed us to combine RFLP and DNA techniques to identify the X chromosome with the mutation responsible for XLA in this family. The finding that II-6 has inherited the same haplotype (A, B) as her carrier sister (II-1), without showing the g  $\rightarrow$  a mutation in the amplification mutagenesis experiments (not shown) suggests that the genomic point mutation most likely originated in the germline of I-1 or I-2.

The SH3 domain is a small molecular domain present in a large number of proteins involved in signal transduction and membrane-cytoskeletal interactions. Whereas the function of the SH3 domain remains unclear, recent reports provided some important insights. Two-hybrid expression cloning was used to identify the SH3 binding protein-1 and to characterize a minimal proline-rich motif within this protein responsible for SH3 binding (18, 19). More recently, a large number of SH3 binding proteins have been identified (reviewed in 47). The critical function of SH3 domains may be the targeting of signaling molecules to specific subcellular locations (48). This would likely allow secondary protein-protein interactions to occur and provide specificity in signal transduction. The biologic importance of SH3 is exemplified by the reported anatomic abnormalities observed in *Caenorhabditis elegans* caused by mutations within SH3 of *sem-5*, a cell signaling gene involved in vulval development and in sex myoblast migration (49).

The presence of a functional SH3 domain is essential for the enzymatic activity of tyrosine kinases. SH3 is often accompanied by another modular domain, SH2, that binds phosphorytyrosine. The crystal structure has now been determined for several SH3 domains (21–26) which form a compact  $\beta$  barrel composed of two  $\beta$  sheets, each formed in turn by a series of two or three  $\beta$  strands (21). The conserved aliphatic and aromatic residues form a hydrophilic binding pocket on



the molecular surface predicted to be the binding site for target proteins. In the BTK of the XLA patients described, the most COOH-terminal of the two exons forming the SH3 domain is deleted. The resulting loss of 21 amino acids, most of which are highly conserved within the BTK and Src families, is predicted to remove two of the five  $\beta$  strands, one from each of the two  $\beta$  sheets, forming the SH3 domain  $\beta$  barrel. In addition, at least three residues (equivalent to Tyr-131, Asn-135, Tyr-136 of c-Src), predicted to reside within the binding pocket of c-Src, are lost (21). As our modeling studies suggest, the deletion of part of SH3 may cause a significant alteration in the folding of the mutant SH3 domain, which could have a dramatic effect on BTK SH3 function. Binding studies with this and other mutated BTK SH3 domains may allow identification of molecules which interact with BTK.

In BTK, SH3 is 5' of SH2 and there are only few residues between them. Deletion of the COOH-terminal part of SH3 and the connecting amino acids could change their spatial relationship. Our modeling suggests that this is not the case, since the distances between the termini are very similar in the wild-type and in the mutated BTK SH3. Thus, it is possible that the mutant protein has an overall structure that is very similar to the wild type differing only by the conformation of the SH3 domain and its connection to SH2. This is supported by the enzymatic activity of the mutant BTK indicating that at least the catalytic domain is properly folded.

Src is negatively regulated by phosphorylation of Tyr-527 in its COOH terminus and binding of this phosphotyrosine

residue to the SH2 domain. The SH3 domain may contribute to the maintenance of an inactive folded Src molecule by stabilizing the SH2 COOH-terminal interaction (50). Deletion of the SH3 domain of c-Src results in marked increase in auto- and transkinase activity and an increase in transforming potential (51-53). In contrast, the normal level of auto- and transkinase activity in the patients described here suggests that similar SH3 mutations in BTK are not upactivating and also are unlikely to result in an increase in transforming potential. The lack of an equivalent COOH-terminal tyrosine in BTK further suggests that BTK is likely to be regulated in alternative ways. Determination of regulatory mechanisms of BTK will be important for understanding its overall function and its role in B cell development.

Although the SH3 deletion described here does not interfere with ATP binding and protein kinase activity, the affected patients from this family have, nevertheless, a severe B cell defect characteristic for XLA. Similar to other cytoplasmic tyrosine kinases, BTK is likely to be involved in signal transduction through interaction with a variety of substrates involved in pathways modulating pre-B and B cell growth, differentiation, and mature cell function. We postulate that the deletion of 21 amino acids within the BTK SH3 domain alters the protein structure so that it disturbs interaction with one or more crucial SH3 binding proteins. This is likely to interrupt the cytoplasmic signal transduction process required for B cell differentiation.

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Address correspondence to Dr. Hans D. Ochs, Department of Pediatrics, RD-20, University of Washington School of Medicine, Seattle, WA 98195.

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